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Phenotypic and genotypic characterization of antimicrobial resistances reveals the effect of the production chain in reducing resistant lactic acid bacteria in an artisanal raw ewe milk PDO cheese

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ABSTRACT

Antimicrobial resistance (AMR) is a significant public health threat, with the food production chain, and, specifically, fermented products, as a potential vehicle for dissemination. However, information about dairy products, especially raw ewe milk cheeses, is limited. The present study analysed, for the first time, the occurrence of AMRs related to lactic acid bacteria (LAB) along a raw ewe milk cheese production chain for the most common antimicrobial agents used on farms (dihydrostreptomycin, benzylpenicillin, amoxicillin and polymyxin B). More than 200 LAB isolates were obtained and identified by Sanger sequencing (V1-V3 16S rRNA regions); these isolates included 8 LAB genera and 21 species. Significant differences in LAB composition were observed throughout the production chain ($P \leq 0.001$), with *Enterococcus* (e.g., *E. hirae* and *E. faecalis*) and *Bacillus* (e.g., *B. thuringiensis* and *B. cereus*) predominating in ovine faeces and raw ewe milk, respectively, along with *Lactococcus* (*L. lactis*) in whey and fresh cheeses, while *Lactobacillus* and *Lacticaseibacillus* species (e.g., *Lactobacillus* sp. and *L. paracasei*) prevailed in ripened cheeses. Phenotypically, by broth microdilution, *Lactococcus, Enterococcus* and *Bacillus* species presented the greatest resistance rates (on average, 78.2 %, 56.8 % and 53.4 %, respectively), specifically against polymyxin B, and were more susceptible to dihydrostreptomycin. Conversely, *Lacticaseibacillus* and *Lactobacillus* were more susceptible to all antimicrobials tested (31.4 % and 39.1 %, respectively). Thus, resistance patterns and multidrug resistance were reduced along the production chain ($P \leq 0.05$). Genotypically, through HT-qPCR, 31 antimicrobial resistance genes (ARGs) and 6 mobile genetic elements (MGEs) were detected, predominating *Str*, *Str*B and *aad*A-01, related to aminoglycoside resistance, and the transposons *tnp*A-02 and *tnp*A-01. In general, a significant reduction in ARGs and MGEs abundances was also observed throughout the production chain ($P \leq 0.001$). The current findings indicate that LAB dynamics throughout the raw ewe milk cheese production chain facilitated a reduction in AMRs, which has not been reported to date.

1. Introduction

Antibiotics are chemical compounds that attack essential bacterial physiology and biochemistry to cause cell death or growth cessation (Lade & Kim, 2021). For decades, antibiotics have been overused, both in human medicine and in animal production (Sobierajski et al., 2022), including the currently forbidden use of subtherapeutic doses as growth promoters (Patel et al., 2020). As a result, bacterial communities have been exposed to antibiotics and have developed the ability to withstand or resist the action of one or more antimicrobial agents, which is called

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antimicrobial resistance (AMR) (Konopka et al., 2022; Virto et al., 2022). Bacteria can be intrinsically resistant to certain antimicrobial groups or agents, mediated by chromosomal genes and linked to physiological or anatomical characteristics. Nonetheless, acquired resistance also occurs due to horizontal transmission between bacteria by means of mobile genetic elements (MGEs), which can carry one or more resistance genes (Iskandar et al., 2022; Nunziata et al., 2022), or due to generational genetic transmission by point mutations in genes that give rise to resistance or increased expression of resistance mechanisms (vertical transmission) (Iskandar et al., 2022; Wall et al., 2016).

The antibiotics utilized in human medicine belong to the same pharmacological classes as those used in veterinary medicine (Devirgiliis et al., 2011); consequently, acquired resistance to certain antimicrobial agents is widespread to the point that effective treatment of certain fatal infections is already compromised (Virto et al., 2022). In fact, the proliferation of antimicrobial-resistant (AR) microorganisms has become one of the most important threats to human health (Wang et al., 2022) and is classified as one of the top 10 threats to global public health (WHO, 2022). It causes approximately 700,000 deaths worldwide per year and is projected to increase to 10 million each year by 2050 (IACG, 2019). Thus, AMR is of utmost importance and is included within the sustainable development goals (SDGs) set by the United Nations. Specifically, AMR affects SDG 3 on good health and well-being since it hinders the ability to control infectious diseases, increasing morbidity and mortality and resulting health care costs (United Nations, 2015).

The food and food production chain is classified as a possible vehicle for the dissemination of AR bacteria and genes (Caniça et al., 2019); and, specifically, fermented products are considered notable reservoirs (Wang et al., 2006; Yasir et al., 2022). In this regard, several studies have been developed recently (Zhao et al., 2022), for instance, on raw beef, sheep and lamb meat (Şanlıbaba, 2022) and dry-fermented sausages (Fraqueza, 2015). However, information about dairy products, especially raw milk cheeses, is limited, with most studies focused on raw cow milk cheeses (Dos Santos et al., 2022; Rola et al., 2016) and scarce information on raw sheep milk cheeses (Gaglio et al., 2016; Slyvka et al., 2022). Milk is an ideal growth medium for microorganisms due to its high nutrient content (Fusco et al., 2020). Consequently, the microbiota of raw ewe milk is diverse and is primarily composed of lactic acid bacteria (LAB), psychotropic bacteria and pathogens (Biçer et al., 2021; Santamarina-García et al., 2022a). Nonetheless, the cheese-making and ripening processes have a clear impact on bacterial communities, with a general predominance of LAB (Cardinali et al., 2021; Santamarina-García et al., 2022a). Several studies have highlighted the presence of resistant bacteria in raw ewe milk and cheese, including pathogenic *Escherichia coli* and *Staphylococcus aureus* (Imre et al., 2022; Karahutová & Bujňáková, 2023; Výrostková et al., 2020, 2021). Nonetheless, despite the predominance of LAB (Quigley et al., 2013; Santamarina-García et al., 2022a), there has been limited research on AMRs in LAB from raw ewe milk and derivate cheeses (Výrostková et al., 2020, 2021). In particular, species of the genus *Enterococcus,* such as *E. faecium* and *E. faecalis*, known as important opportunistic pathogens in nosocomial infections (Conde-Estévez et al., 2011), have been described as the most remarkable AR LAB (Výrostková et al., 2021). Addressing AMRs in LAB is essential since they can serve as potential reservoirs for the transfer of resistance genes to other bacteria, including pathogenic bacteria (Caniça et al., 2019).

Several studies have reported the preference of consumers for raw milk cheeses (Colonna et al., 2011; Meunier-Goddik & Waite-Cusic, 2019), based on their richer and more intense aromatic profiles than pasteurized milk cheeses (Barron et al., 2007; O'Sullivan & Cotter, 2017). Given the pressing need to minimize the development and dissemination of AR LAB to safeguard public health (Výrostková et al., 2021), the present study is focused on Idiazabal protected designation of origin (PDO) cheese. It is a semihard or hard cheese from the Basque Country (southwestern Europe) produced with raw milk from the Latxa and/or Carranzana autochthonous sheep breeds, and it has a minimum mandatory ripening period of 60 days (Official Journal of the European Communities, 1996). Thus, this study aimed to characterize the prevalence of AMRs in LAB from ovine faeces, raw ewe milk, whey, fresh cheeses and 2-month-old ripened cheeses by means of phenotypic and genotypic approaches. Moreover, the potential differences among producers producing the same kind of raw ewe milk cheese were also analysed. To our knowledge, no study has comprehensively analysed the prevalence of AMRs along the production chain of a raw ewe milk cheese.

2. Methods

2.1. Area of study

To evaluate the prevalence of AMRs in LAB along the production chain of artisanal raw ewe milk cheeses, this study was carried out within the European PDO Idiazabal cheese. This particular cheese was selected as a case study because its production is primarily carried out by small-scale artisanal dairies that oversee the entire process, from herd management to cheese-making. Idiazabal cheese is a semihard or hard cheese made from the raw milk of the autochthonous Latxa and/or Carranzana sheep breeds and has a mandatory minimum ripening time of 2 months. Herd management and milk production for cheese-making occur in the Basque Country, covering an area of 17,213.06 km^2 in southwestern Europe (43° 27′ − 41° 54′ N and 1° 5′ − 3° 37′ W). This region corresponds to the natural habitat of the sheep breeds (Official Journal of the European Communities, 1996). Herd management involves the use of indoor forage from October to March and semi-extensive or extensive grazing from March to October (Aldalur et al., 2019). Milk collection and cheese production mainly occur between January and June, following the traditional seasonal approach dictated by the biological rhythms of the sheep (Boletín Oficial del Estado, 1993).

2.2. Sampling

For sampling, four producers attached to the PDO Idiazabal cheese were chosen and identified as A, B, C, and D. Each producer came from one of the distinct geographical production areas (Alava, Biscay, Gipuzkoa, and Navarre). All the producers adhered to similar flock management and cheese-making practices in accordance with the specifications outlined by the Idiazabal PDO regulatory board (Boletín Oficial del Estado, 1993). The flocks consisted of approximately 350–400 Latxa breed sheep, following the management practices mentioned earlier. Milking was conducted automatically, and the milk was promptly refrigerated (3–4 °C) until cheese-making. For the cheese-making process, the milk was warmed to 25 °C, and the commercial mesophilic lyophilized starter culture Choozit MM 100 LYO 50 DCU (a mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, DuPont NHIB Ibérica S.L., Barcelona, Spain) was added. Coagulation occurred at 28–32 °C for 20–45 min using artisanal rennet and/or the commercial NATUREN® 195 Premium (Chr. Hansen Holding A/S, Hørsholm, Denmark). The resulting curds were cut into 5–10 mm diameter grains and heated to 36–38 °C. Cheeses were then moulded, pressed and salted in saturated brine, and subsequently ripened in chambers maintained at 80–95 % relative humidity and 8–14 °C for 2 months. Thus, ovine faeces, raw ewe milk, whey, fresh cheeses (1-day-old), and 2-month-old ripened cheese samples were obtained from each producer. Samples were collected aseptically in quadruplicate, with each set of samples corresponding to the same batch. The sampling was conducted by the producers, eliminating the need for approval from the Ethics Committee for Animal Experimentation. Verbal consent was obtained from dairies during samples collection. Samples were collected from healthy flocks, excluding animals that underwent antibiotic treatment. Samples were transported under refrigerated conditions (3 ± 1 °C) for analysis.

2.3. Reagents and materials

The peptone water was supplied by Panreac Química (Barcelona, Spain). De Man, Rogosa and Sharpe (MRS) agar, MRS broth medium, sodium citrate and sodium chloride were purchased from Scharlab (Barcelona, Spain). Tryptic soy broth (TSB) was obtained from Condalab (Madrid, Spain). Glycerol was obtained from Honeywell Fluka (Madrid, Spain). Amoxicillin was supplied by Sigma-Aldrich (Madrid, Spain). Dihydrostreptomycin and polymyxin B were purchased from Glentham Life Sciences (Corsham, United Kingdom). Benzylpenicillin was supplied by Tokyo Chemical Industry Co. (Tokyo, Japan). Mag-Bind Bacterial DNA 96 Kit was purchased from Omega Bio-Tek, Inc. (Norcross, United States). KAPA HiFi HotStart ReadyMix Kit was obtained from Roche Molecular Systems, Inc. (Branchburg, United States). CleanNGS and CleanDTR kits were obtained from CleanNA (Waddinxveen, The Netherlands). DNA 5 K Reagent Kit was obtained from PerkinElmer, Inc. (Waltham, United States). BigDye Terminator v3.1 Cycle Sequencing Kit and exonuclease I were purchased from Thermo Scientific (Waltham, United States). QIAamp® PowerFecal® Pro DNA Kit and QIAGEN® Multiplex PCR Kit were purchased from Qiagen (Valencia, United States). Petri dishes and 96-well plates were obtained from Deltalab (Barcelona, Spain). The Master Mix SsoFastTM EvaGreen® Supermix Kit with Low ROX was purchased from Bio-Rad Laboratories (Hercules, United States).

2.4. Phenotypic characterization of AMRs

2.4.1. LAB isolation and enumeration

For the faeces and cheese samples, 10 g was diluted in duplicate 1:10 in peptone water and homogenized for 30 s three times in a stomacher (Masticator Basic 400, IUL Instruments, Königswinter, Germany). Serial dilutions were made in peptone water and plated on MRS agar media. For the raw ewe milk and whey samples, 100 μL was taken directly and serially diluted. The plates were incubated at 37 ± 1 °C for 48 h. Three presumptive LAB isolates were randomly selected per sample based on colony morphology diversity. Then, the isolates were preenriched in TSB and subcultured onto MRS agar to ensure purity. All the cultures were stored at − 80 °C in 20 % (v/v) glycerol.

2.4.2. Identification of LAB isolates by Sanger sequencing

2.4.2.1. DNA extraction. Isolates were preenriched in TSB and subcultured onto MRS agar prior to DNA extraction to ensure purity and viability. Bacterial DNA was extracted using the Mag-Bind Bacterial DNA 96 Kit following the manufacturer's instructions for agar cultures, but the elution volume was reduced to 60 μL to improve the DNA yield. The quantity and quality of the DNA obtained were verified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Massachusetts, USA), in which the absorbance was measured at a wavelength of 260 nm and the 260/280 and 260/230 ratios were analysed. DNA extraction and subsequent Sanger sequencing were conducted in the Sequencing and Genotyping Unit of the Genomic Facility/SGIker (supported by UPV/EHU, MICINN, GV/EJ, FSE) of the University of the Basque Country.

2.4.2.2. Sanger sequencing. The V1–V3 regions of the 16S rRNA gene were amplified via PCR with the KAPA HiFi HotStart ReadyMix Kit using the forward primer 16S–V1–8F: 5′- AGAGTTTGSTCCTGGCTCAG-3′ and the reverse primer 16S–V3–534R: 5′- ATTACCGCGGCTGCTGG −3′. The PCR products were purified by means of the CleanNGS Kit following the manufacturer's instructions. Amplicon quantification was performed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and a LabChip GX Touch Nucleic Acid Analyser (PerkinElmer) with a DNA 5 K Reagent Kit. Sequencing of the purified product was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit following the manufacturer's protocol. The sequencing product was purified using a magnetic bead-based CleanDTR kit, and Sanger sequencing was performed on the SeqStudio platform (Thermo Fisher).

2.4.2.3. Bioinformatic analysis. Quality filtering and trimming of the raw reads were performed using SeqStudio Reporter software (Thermo Scientific). The sequences were visualized and edited by means of the BioEdit Sequence Alignment Editor software 7.2.5 (Hall et al., 2011). The resulting sequences were approximately 500 bp in length. Taxonomic classification was performed against the Nucleotide Basic Local Alignment Search Tool (NBLAST) 2.14.0+ (Zhang et al., 2000), with default parameters and taking into account the e-value, score, query cover and percentage of identification as quality indicators.

2.4.3. Antimicrobial susceptibility testing (AST) via the broth microdilution method

The minimum inhibitory concentration (MIC) of the LAB isolates was evaluated by the broth microdilution method for the most widely used antimicrobial agents on farms (namely, amoxicillin, dihydrostreptomycin, benzylpenicillin and polymyxin B) according to the updated International Organization for Standardization and International Dairy Federation Standards (ISO/IDF, 2010) and European Food Safety Authority guidance (Rychen et al., 2018), with minor modifications. Briefly, a 96-well plate was inoculated with MRS broth medium supplemented with serial (1:2) concentrations of antibiotics (amoxicillin: 0.0313–16 μg/mL; dihydrostreptomycin: 1–512 μg/mL; benzylpenicillin: 0.0313–32 μg/mL; and polymyxin B: 2–1024 μg/mL). The inocula of each isolate were prepared in saline solution (0.85 %, m/v) by picking up single colonies from previously subcultured isolates on MRS agar to obtain an optical density equivalent to 0.5 on the MacFarland scale. The inoculum was subsequently diluted 1:10 in antibiotic-free MRS broth, and 50 μL of the diluted suspension was added to each well and incubated at 37 ± 1 °C for 48 h. The inoculum in a well with MRS broth without antibiotics was used as a positive control, and an inoculum-free well was used as a negative control. The antimicrobial susceptibility or resistance was interpreted using the available microbiological cut-off values defined by the European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (Rychen et al., 2018) and employing the epidemiological cut-off values (ECOFFs) proposed by the European Committee for Antimicrobial Susceptibility Testing (EUCAST; https://www.eucast.org).

2.5. Genotypic characterization of AMRs

2.5.1. DNA extraction

To analyse the presence of antimicrobial resistance genes (ARGs), DNA was extracted as previously described (Santamarina-García et al., 2022a), with some modifications. Briefly, for the faecal and cheese samples, 10 g was suspended in 90 mL of 2 % (w/v) sterile sodium citrate (pH 8.0) and homogenized six times (20 s ON and 10 s OFF) in a stomacher (Masticator Basic 400; IUL Instruments, Königswinter, Germany). The resulting suspension was centrifuged at 6500 × g for 8 min at 4 °C, after which the fat-containing supernatant was discarded. The obtained pellet was washed with 50 mL of sodium citrate and centrifuged at 6500 × g for 8 min at 4 °C. The pellet was resuspended in 800 μL of sodium citrate and centrifuged three times at 6500 × g for 8 min at 4 °C. The DNA was extracted with a QIAamp® PowerFecal® Pro DNA Kit according to the manufacturer's protocol, but a double DNA elution step was carried out with 25 μL of C6 solution to improve DNA yields. To extract DNA from the milk and whey samples, 10 mL was processed as described above, but without the need for homogenization in the stomacher. The DNA was stored at − 80 °C until analysis.

2.5.2. High-throughput quantitative PCR (HT-qPCR)

The detection of ARGs was performed by means of HT-qPCR in a nanofluidic qPCR BioMarkTM HD system using 96.96 Dynamic Array Integrated Fluidic Circuits (IFCs) (Fluidigm Corporation), as previously described (Jauregi et al., 2021). A total of 48 primer sets were used (Supplementary Table 1) to target the ARGs conferring resistance against the most commonly used antimicrobial agents on farms (12 ARGs encoding resistance to dihydrostreptomycin, 24 ARGs for benzylpenicillin and amoxicillin, 2 ARGs for polymyxin B and 2 multidrug ARGs conferring resistance to more than one of the aforementioned antimicrobial agents), MGE genes (5 genes encoding transposases and 2 genes encoding integrases) and the 16S rRNA gene as a reference gene. These genes were selected considering the CARD database for LAB (Alcock et al., 2023). The primers used for qPCR were previously validated (Gorecki et al., 2022; Hu et al., 2016). DNA samples were pre-amplified using the QIAGEN® Multiplex PCR Kit and a primer pool (final concentration for each primer pair = 50 nM), following the amplification program (at 95 °C for 15 min and 14 PCR cycles at 95 °C for 15 s, 60 °C for 4 min and a final extension step at 4 °C). Then, the samples were treated with exonuclease I (at 37 °C for 30 min for digestion, 80 °C for 15 min for inactivation of exonuclease I and kept at 4 °C). Subsequently, 1:10 dilutions of specific target amplification reactions were loaded onto the Dynamic Array IFCs following the Fluidigm's Fast Gene Expression Analysis—EvaGreen® Protocol (Fluidigm Corporation). For amplification, the Master Mix SsoFastTM EvaGreen® Supermix Kit with Low ROX was used, with a final concentration of primers of 500 nM, both forward and reverse. The program consisted of 1 min of denaturation at 95 °C, followed by 30 cycles of 95 °C for 5 s and 60 °C for 20 s, a melting curve at 60 °C for 3 s and a ramp rate of 1 °C/3 s up to 95 °C. Four replicates were included for each sample. Analyses were conducted at the Gene Expression Unit of The Genomics Facility/SGIker (supported by UPV/EHU, MICINN, GV/EJ, FSE) of the University of the Basque Country.

2.5.3. Bioinformatic analysis

Raw data were processed with Fluidigm Real-Time PCR Analysis Software (v.3.1.3, Fluidigm Corporation), with linear baseline correction and manual threshold settings. A cycle threshold (CT) value of 30 was chosen because the highest CT value obtained in this study was 29.0. The detection of an ARG or MGE gene was considered positive when 3 out of the 4 technical replicates for each sample were above the detection limit. The relative abundances of the ARGs were calculated on the basis of the comparative CT method (Jauregi et al., 2021), normalized to the abundance of the 16S rRNA control gene and expressed as the fold change (FC).

$$\Delta CT(per\ replicate) = CT(target\ gene) - CT(16S\ rRNA\ gene)$$

$$\Delta\Delta CT(per\ sample) = \overline{\Delta CT}$$

$$FC = 2^{-\Delta\Delta CT}$$

2.6. Statistical analysis

IBM SPSS statistical package version 26.0 (IBM SPSS, Inc., Chicago, IL, USA, 2019) was used for data preparation and analysis. Plot generation was performed in RStudio version 2023.03.1 and R version 4.3.0 (R Core Team, Vienna, Austria, 2023) with the "ggplot2" package (https://github.com/tidyverse/ggplot2) and in Microsoft Office Professional Plus 2016 Excel® version 16.0.5413 (Microsoft, Albuquerque, United States). Kruskal–Wallis one-way analysis of variance (ANOVA) with Bonferroni correction was performed with the SPSS package to determine the significance ($P \leq 0.05$) of the effects of producer and production chain (sample type) factors on the bacterial counts and abundances and phenotypic and genotypic results. Permutational multivariate analysis of variance (PERMANOVA) was carried out in R with the "vegan" package (https://github.com/vegandevs/vegan) to analyse the overall effect of producer and production chain factors. The data were log transformed when necessary and subjected to unit variance (UV) scaling, and a heatmap with hierarchical clustering analysis (HCA) was generated with the "pheatmap" package (https://github.com/raivokolde/pheatmap) to analyse the clustering of the phenotypic and genotypic results. Clustering of samples according to phenotypic and genotypic results was also performed by means of a dendrogram in R with the "factoextra" package (https://github.com/kassambara/factoextra). Trends in the bacterial counts and abundances and phenotypic and genotypic results according to producer and production chain factors were explored by means of principal component analysis (PCA), applied to log-transformed, when necessary, and UV-scaled data and performed in SIMCA software version 17.0.2.34594 (Umetrics AB, Umeå, Sweden). The number of principal components (PCs) was determined by the eigenvalues (greater than 1.0) and cross-validation. Similarly, orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed with SIMCA software to analyse whether the samples differed according to the producer and production chain factors. Variable influence on projection (VIP) values and loading weights were used to analyse the importance of each parameter in the model.

3. Results

3.1. LAB prevalence and distribution throughout the cheese production chain

Fig. 1A shows the prevalence of LAB throughout the Idiazabal cheese production chain. Overall, large differences were found according to the sample type ($P \leq 0.001$). Specifically, a mean LAB prevalence of 6.45 ± 0.451 log CFU/g was observed in the faeces. In the raw ewe milk, the LAB count was 3.73 ± 0.0659 log CFU/mL, which subsequently increased to 5.65 ± 0.0623 log CFU/mL in the whey and to 7.99 ± 0.172 log CFU/g in the fresh cheeses. However, during ripening, the LAB count slightly decreased to 7.75 ± 0.202 log CFU/g, although the difference was not significant (Fig. 1A). Moreover, among producers, significant differences were also observed for the whey ($P \leq 0.01$) and fresh cheese samples ($P \leq 0.05$), with producer A clearly differentiated from the rest due to the lower values. Using multivariate analysis, PERMANOVA confirmed the differences among sample types ($P \leq 0.001$) and, to a lesser extent, among producers ($P \leq 0.05$).

To identify the LAB communities, 203 isolates were obtained from the raw ewe milk Idiazabal cheese production chain. As expected, all the isolates belonged to the phylum Firmicutes and class Bacilli (Fig. 1B). Two orders were identified, predominantly Lactobacillales (69.0 %) and, to a lesser extent, Bacillales (31.0 %). All the isolates of the Bacillales order belonged to the *Bacillaceae* family and the *Bacillus* genus, identifying 4 different species, *B. cereus* (8.37 %), *B. thuringiensis* (6.40 %), *B. paramycoides* (2.96 %), and *B. anthracis* (0.99 %), in addition to other unidentified species (*Bacillus* sp., 12.3 %). The isolates of the order Lactobacillales belonged mainly to the *Enterococcaceae* (37.4 %) and *Lactobacillaceae* (22.2 %) families and, to a lesser extent, to the *Streptococcaceae* (9.36 %). All the *Enterococcaceae* isolates corresponded to the genus *Enterococcus*, identifying different species, such as *E. hirae* (19.2 %) and *E. faecalis* (13.3 %), and to a lesser extent, *E. faecium* (2.46 %), *E. mundtii* (1.48 %), *E. avium* (0.49 %) and *E. durans* (0.49 %). The *Lactobacillaceae* isolates belonged to the genus *Lactobacillus*, without being able to identify species (*Lactobacillus* sp., 7.88 %); *Lacticaseibacillus* (7.88 %), with the species *L. paracasei* (6.40 %) and *L. casei* (1.48 %); *Levilactobacillus* (3.94 %), namely, *L. brevis*; and *Lactiplantibacillus* (2.46 %), identified as *L. plantarum* (0.49 %) and *L. plantarum* subsp. *plantarum* (1.97 %). The *Streptococcaceae* isolates belonged to the genera *Lactococcus* (8.87 %), identifying *L. lactis* (4.43 %) and *L. lactis* subsp. *lactis* (3.45 %), in addition to unidentified strains (0.99 %), and *Streptococcus*, for which species could not be identified (*Streptococcus* sp.,

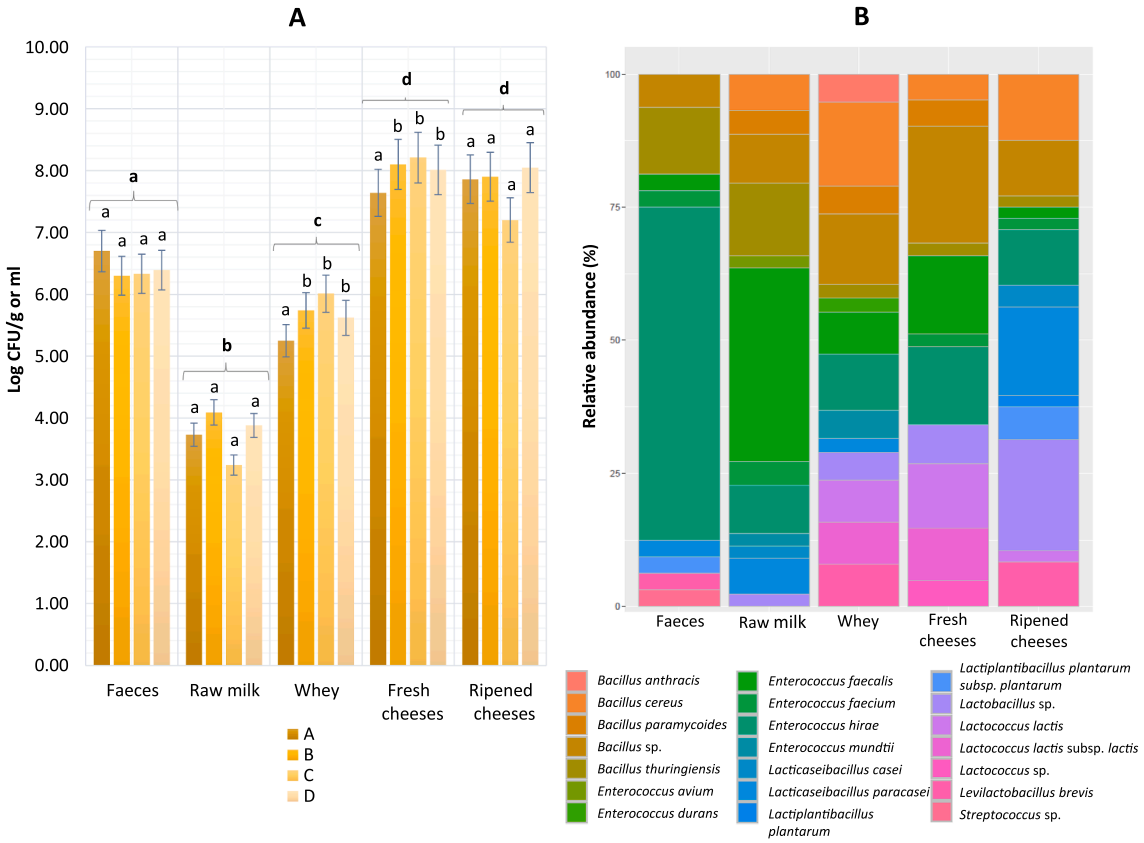

Fig. 1. Mean counts (log CFU/g or mL) and relative abundance (%) of lactic acid bacteria throughout the Idiazabal cheese production chain (faeces, raw milk, whey, fresh cheese and ripened cheese samples). The different lowercase letters for each type of sample indicate statistically significant differences.

0.49 %). Within all the samples, *E. hirae, E. faecalis, Bacillus* sp., *B. cereus* and *Lactobacillus* sp. were some of the most important species throughout the production chain of the Idiazabal cheese.

PERMANOVA confirmed the difference in LAB composition among the collected samples throughout the production chain ($P \leq 0.001$) (Fig. 1B). *E. hirae* clearly predominated in the faeces (62.5 %), followed by *B. thuringiensis* (12.5 %) and other unidentified species (*Bacillus* sp.) (6.25 %). In raw ewe milk, instead, *E. faecalis* (36.4 %) predominated, followed by *B. thuringiensis* (13.6 %), *Bacillus* sp. (9.09 %) and *E. hirae* (9.09 %). During cheese-making, *Bacillus* species, such as *B. cereus* (15.8 %) or *Bacillus* sp. (13.2 %), dominated the whey; together with *Enterococcus*, such as *E. hirae* (10.5 %) or *E. faecalis* (7.89 %); and *Lactococcus*, *L. lactis* (7.89 %) and *L. lactis* subsp. *lactis* (7.89 %), or *L. brevis* (7.89 %). In fresh cheeses, a similar trend was maintained, with a predominance of unidentified *Bacillus* species (22.0 %), along with *Enterococcus species*, such as *E. hirae* (14.6 %) and *E. faecalis* (14.6 %), and also *L. lactis* (12.2 %). However, after ripening, *Lactobacillus* species predominated (20.8 %), followed by *Lacticaseibacillus*, specifically *L. paracasei* (16.7 %). In general, the abundance of other species, such as *E. hirae* (10.4 %) and *Bacillus* sp. (10.4 %), decreased during ripening. The greatest differences among sample types along the production chain were mainly observed for *E. faecalis, L. paracasei, L. lactis* and *Lactobacillus* sp. ($P \leq 0.05$). PERMANOVA corroborated the lack of differentiation among producers ($P > 0.05$).

3.2. Phenotypic profile of antimicrobial resistance

Subsequently, antimicrobial susceptibility was tested by the broth microdilution method for more than 200 LAB isolates. The distributions of MICs are shown in Table 1. Clear differences were observed in the AMR phenotypes among the LAB communities ($P \leq 0.05$) (Table 1 and Fig. 2A), which was confirmed by an OPLS-DA model (Supplementary Fig. 1). Overall, *Lactococcus* and *Streptococcus* species had the greatest resistance rates (on average, 78.2 % and 75.0 % of resistance of all isolates to all antibiotics, respectively), followed by *Levilactobacillus, Enterococcus* and *Bacillus* (65.6 %, 56.8 % and 53.4 %, respectively). *Lactiplantibacillus, Lacticaseibacillus* and *Lactobacillus* species, instead, were the most susceptible bacteria (31.3 %, 31.4 % and 39.1 %, respectively).

In more detail, clear differences were observed in the AMR phenotypes among LAB species from the same genera and families ($P \leq 0.05$). Within the *Bacillaceae* and *Bacillus* genus, *B. anthracis* species clearly differed from the other species because of their low resistance (12.5 %), with the remaining species, *B. cereus*, *B. paramycoides*, *B. thuringiensis* and *Bacillus* sp., exhibiting greater resistance (69.1 %, 58.3 %, 71.2 % and 56.0 %, respectively). For the *Enterococcaceae* and *Enterococcus* isolates, differences were also detected among the species, with *E. durans* and *E. faecium* being the most resistant (100 % and 80.0 %, respectively); *E. hirae, E. mundtii* and *E. faecalis* presenting greater susceptibility (57.1 %, 58.3 % and 45.4 %, respectively); and *E. avium* isolates being sensitive to all the antibiotics tested. On the other hand, most *Lactobacillaceae* genera and species showed similar low resistance rates, including unidentified *Lactobacillus* species (39.1 %), *Lacticaseibacillus* species, namely, *L. paracasei* and *L. casei* (21.2 % and 41.7 %, respectively), and *Lactiplantibacillus* species, specifically *L. plantarum* and *L. plantarum* subsp. *plantarum* (25.0 % and 37.5 %, respectively). The *Levilactobacillus* genus and the *L. brevis* species were unique exceptions for their higher levels of resistance. Finally, *Streptococcaceae* isolates also showed low differences among genera and species. All *Lactococcus* species, including *L. lactis*, *L. lactis* subsp. *lactis* and other unidentified species (72.2 %, 75.0 % and 88.0 % on average, respectively), exhibited high resistance, similar to unidentified *Streptococcus* species (75.0 % on average). Thus, the HCA and dendrogram divided the LAB communities into two clusters (Fig. 2A and B). *E. avium, L. plantarum, L. plantarum* subsp. *plantarum* and

Table 1
Distribution of minimum inhibitory concentration (MIC) values for the 202 isolates obtained throughout the production chain (ovine faeces, raw ewe milk, whey, fresh cheese and 60-day-old ripened cheese) of raw ewe milk Idiazabal cheese.

Bacterial species	Antimicrobial compound	MIC (μg/kg)[1]																n	Resistance[2]						MIC_{50} (μg/kg)[3]	MIC_{90} (μg/kg)[4]
		< 0.032	0.032	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024		Total	Faeces	Raw milk	Whey	Fresh cheese	Ripened cheese		
B. anthracis	Dihydrostreptomycin						1			1									2	0.00			0.00			< 1	4
	Polymyxin b							1				1							2	50.0	n.d.	n.d.	50.0	n.d.	n.d.	< 2	16
	Benzylpenicillin		2																2	0.00			0.00			0	0
	Amoxicillin	1	1																2	0.00			0.00			< 0.032	0
B. cereus	Dihydrostreptomycin						5	2	2	1	1		1			1	4		17	35.3		100	33.3	0.00	16.7	2	512
	Polymyxin b							1	1	2	2	1	1	2	2	3		2	17	88.2	n.d.	100	100	0.00	100	64	1024
	Benzylpenicillin	1			1		1		2		1	11							17	82.4		100	66.7	50.0	100.0	16	16
	Amoxicillin	5										12							17	70.6		66.7	83.3	0.00	83.3	16	16
B. paramycoides	Dihydrostreptomycin						2		1			1	1				1		6	50.0		50.0	50.0	50.0		2	512
	Polymyxin b							2		1		2				1			6	66.7	n.d.	50.0	50.0	100	n.d.	8	256
	Benzylpenicillin	1			1							4							6	66.7		50.0	50.0	100		16	16
	Amoxicillin	2			1			1				2							6	50.0		50.0	50.0	50.0		0.125	16
B. thuringiensis	Dihydrostreptomycin						1	1	1	1				1	1		6		13	61.5	25.0	66.7	100	100	100	128	512
	Polymyxin b							2		1		1	2	2	1	2		2	13	84.6	75.0	83.3	100	100	100	64	1024
	Benzylpenicillin		1	1	1			2		1		7							13	76.9	75.0	83.3	100	0.00	100	16	16
	Amoxicillin	4			1		1					7							13	61.5	100.0	66.7	0.00	0.00	0.00	16	16
Bacillus sp.	Dihydrostreptomycin						10	2	2	1	1						9		25	36.0	50.0	50.0	60.0	33.3	0.00	2	512
	Polymyxin b							8				1	1	1	4	4	1	5	25	68.0	50.0	75.0	100	33.3	100	128	1024
	Benzylpenicillin	5	1	1			1					17							25	68.0	50.0	50.0	100	44.4	100	16	16
	Amoxicillin	7	2		3							13							25	52.0	50.0	50.0	80.0	33.3	60.0	16	16
E. avium	Dihydrostreptomycin						1												1	0.00		0.00				< 1	< 1
	Polymyxin b							1											1	0.00	n.d.	0.00				2	2
	Benzylpenicillin	1																	1	0.00		0.00	n.d.	n.d.	n.d.	< 0.032	< 0.032
	Amoxicillin	1																	1	0.00		0.00				< 0.032	< 0.032
E. durans	Dihydrostreptomycin																1		1	100			100			512	512
	Polymyxin b														1				1	100	n.d.	n.d.	100	n.d.	n.d.	128	128
	Benzylpenicillin											1							1	100			100			16	16
	Amoxicillin											1							1	100			100			16	16
E. faecalis	Dihydrostreptomycin						3	3	2		1	2	2	1	2	1	10		27	40.7	0	43.8	33.3	50.0	0.00	64	512
	Polymyxin b							8	1		2	2	4	1	4			5	27	66.7	100	68.8	33.3	83.3	0.00	32	1024
	Benzylpenicillin	3	3	4		1	1	1	1	1	4	8							27	29.6	100	37.5	0.00	16.7	0.00	2	16
	Amoxicillin	9	1	1	1	1			1	1		12							27	44.4	0	62.5	33.3	16.7	0.00	2	16
E. faecium	Dihydrostreptomycin						1										4		5	80.0	0.00	100		100	100	512	512
	Polymyxin b							1				2				1		1	5	80.0	0.00	100	n.d.	100	100	16	1024
	Benzylpenicillin	1										4							5	80.0	0.00	100		100	100	16	16
	Amoxicillin		1									4							5	80.0	0.00	100		100	100	16	16
E. hirae	Dihydrostreptomycin						4	4	4	2	1	2	3	3	1	4	11		39	38.5	35.0	75.0	0.00	16.7	80.0	32	512
	Polymyxin b							4	4	3	2	10	4	1	3	3		5	39	79.5	85.0	100	25.0	66.7	100	16	1024
	Benzylpenicillin	1	1	8	2	2	2	1		1	1	20							39	51.3	50.0	75.0	25.0	33.3	80.0	16	16
	Amoxicillin	10	2	1	1	1				1		23							39	59.0	60.0	75.0	25.0	33.3	100	16	16
E. mundtii	Dihydrostreptomycin						1	1				1							3	0.00		0.00	0.00			1	16
	Polymyxin b							1					1		1				3	66.7	n.d.	100	50.0	n.d.	n.d.	32	128
	Benzylpenicillin				1							2							3	66.7		100	50.0			16	16
	Amoxicillin											3							3	100		100	100			16	16
Lactobacillus sp.	Dihydrostreptomycin							7		1	2	1	1			1	3		16	25.0		0.00	50.0	33.3	20.0	2	512
	Polymyxin b								6	1		1	2		1	2	1	2	16	56.3	n.d.	0.00	100	33.3	60.0	8	1024
	Benzylpenicillin	1	1			1	1	1	1	1	2		7						16	43.8		0.00	50.0	0.00	60.0	4	16
	Amoxicillin	9	1	1									5						16	31.3		0.00	50.0	66.7	20.0	< 0.032	16
L. casei	Dihydrostreptomycin							1						1			1		3	33.3		100			0.00	32	512
	Polymyxin b								1				1			1			3	66.7	n.d.	100	n.d.	n.d.	50.0	16	256
	Benzylpenicillin								1			1	1						3	33.3		0.00			50.0	8	16
	Amoxicillin	1			1								1						3	33.3		100			0.00	0.0625	16
L. paracasei	Dihydrostreptomycin							9				1			1		2		13	23.1	0.00	33	0.00		25.0	< 1	512
	Polymyxin b								9			1	1	1		1			13	30.8	100	33	100	n.d.	12.5	< 2	32
	Benzylpenicillin	7			1		2						3						13	23.1	0.00	0	100		25.0	< 0.032	16
	Amoxicillin	11				1							1						13	7.69	0.00	0	100		0.00	< 0.032	0.125
L. plantarum	Dihydrostreptomycin							1											1	0.00					0	< 1	< 1
	Polymyxin b								1										1	0.00					0	< 2	< 2
	Benzylpenicillin												1						1	100	n.d.	n.d.	n.d.	n.d.	100	16	16
	Amoxicillin	1																	1	0.00					0	< 0.032	< 0.032
L. plantarum subsp. *plantarum*	Dihydrostreptomycin							2		1							1		4	25.0	100				0	< 1	512
	Polymyxin b								1	1			1					1	4	50.0	100		n.d.	n.d.	33	2	1024
	Benzylpenicillin	1				1							2						4	50.0	100				33	0.125	16
	Amoxicillin	2						1					1						4	25.0	100				0	< 0.032	16
L. lactis	Dihydrostreptomycin							2	1	1							5		9	55.6			66.7	60.0	0	512	512
	Polymyxin b								1	1	1				1		1	4	9	77.8	n.d.	n.d.	100	80.0	0	512	1024
	Benzylpenicillin	1			1								7						9	77.8			100	60.0	100	16	16
	Amoxicillin	2											7						9	77.8			100	60.0	100	16	16
L. lactis subs. *lactis*	Dihydrostreptomycin							1		1		1					4		7	57.1			33.3	75.0		512	512
	Polymyxin b												1		1	1		4	7	100	n.d.	n.d.	100	100	n.d.	1024	1024
	Benzylpenicillin	1					1						5						7	71.4			66.7	75.0		16	16
	Amoxicillin	2											5						7	71.4			66.7	75.0		16	16
Lactococcus sp.	Dihydrostreptomycin																2		2	100				100		512	512
	Polymyxin b																	2	2	100	n.d.	n.d.	n.d.	100	n.d.	1024	1024
	Benzylpenicillin												2						2	100				100		16	16
	Amoxicillin	1											1						2	50.0				50.0		< 0.032	16
L. brevis	Dihydrostreptomycin							2				1		1	1		3		8	37.5	0.00		33.3		50.0	32	512
	Polymyxin b								2			1			2	1		2	8	75.0	100	n.d.	66.7	n.d.	75.0	64	1024
	Benzylpenicillin	1	1										6						8	75.0	100		66.7		50.0	16	16
	Amoxicillin	2											6						8	75.0	100		66.7		50.0	16	16
Streptococcus sp.	Dihydrostreptomycin											1							1	0.00	0.00					8	8
	Polymyxin b																1		1	100	100	n.d.	n.d.	n.d.	n.d.	512	512
	Benzylpenicillin												1						1	100	100					16	16
	Amoxicillin												1						1	100	100					16	16

[1]The range of dilutions tested for each antimicrobial agent is indicated in white. The vertical lines indicate the epidemiological cut-off (ECOFF) values according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or European Food Safety Authority (EFSA). MICs lower than the lowest concentration tested are indicated in the closest concentration of the grey range.
[2]n.d. = not detected.
[3] MIC_{50} (μg/kg) = MIC requeried for the inhibition of the growth of the 50% of the isolates.
[4] MIC_{90} (μg/kg) = MIC requeried for the inhibition of the growth of the 90% of the isolates.

B. anthracis were the most differentiated LAB, as they were the most sensitive bacteria to all the antibiotics tested (on average, 18.8 % of all the isolates were resistant to all the antibiotics) (cluster 1). On the other hand, the remaining LAB species exhibited higher resistance rates (cluster 2). *L. casei, L. paracasei, E. faecalis* and *Lactobacillus* sp. were closely related, as they showed higher but still lower resistance rates (on average, 36.8 %) (cluster 2.3). *E. durans, Lactococcus* sp., *E. mundtii* and *Streptococcus* sp. stood out (clusters 2.1 and 2.2), showing the highest resistance rates to all the antimicrobial agents tested (93.8 %). The resistance rate against dihydrostreptomycin was the main reason for the

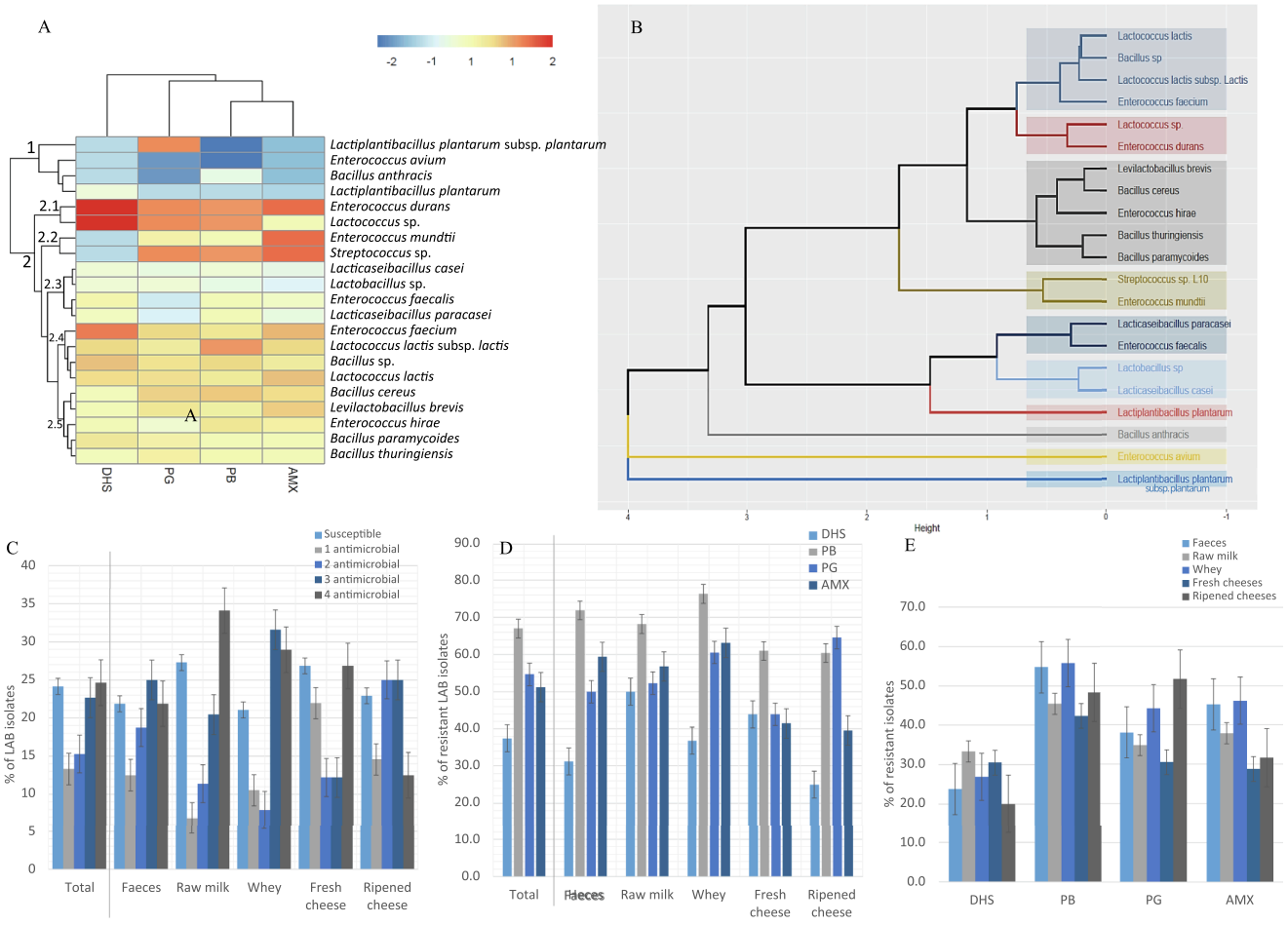

Fig. 2. HCA heatmap (A), dendogram clustering (B), box plot representations (C, D and E) based on antimicrobial susceptibility testing (AST) results according to the bacterial species (A and B), number of antimicrobials (C) and sample type along production chain (D and E). Abbreviations: DHS: dihydrostreptomycin; PB: polymyxin B; PG: benzylpenicillin; AMX: amoxicillin.

difference between these two clusters (2.1 and 2.2). The remaining bacterial species belonging to the *Bacillus, Enterococcus, Lactococcus* or *Levilactobacillus* genera exhibited similar high-intermediate resistance (66.7 % and 67.2 %, respectively) (clusters 2.4 and 2.5).

The MIC_{50} and MIC_{90}, defined as the MIC required for the inhibition of the growth of 50 % and 90 %, respectively, of the isolates confirmed the high resistance of some of the bacterial species (Table 1). However, some trends were observed along the production chain (Supplementary Table 2). *B. cereus, E. hirae, E. mundtti, L. lactis* subsp. *lactis* and *L. brevis* maintained similar MIC_{50} and MIC_{90} values throughout the production chain, while for *B. paramycoides*, *E. faecium*, *L. paracasei* and *Lactobacillus* sp. increased, indicating a greater prevalence of resistant bacteria throughout the production chain. Finally, the MIC_{50} and MIC_{90} of *B. thuringiensis*, *Bacillus* sp., *E. faecalis*, *L. casei*, *L. plantarum* subsp. *plantarum* and *L. lactis* decreased, which indicated an increase in the abundance of sensible bacteria during the cheese production process.

In general, 24.1 % (49/203) of the LAB isolates were susceptible to all the antimicrobial agents tested (Fig. 2C). Thus, 75.9 % (154/203) were resistant to at least one of the antibiotics tested, with resistance to 3 or 4 antimicrobial agents being the most common (46/203, 22.7 % and 50/203, 24.6 %, respectively) (Fig. 2C). However, differences were observed throughout the cheese production chain and were mainly related to differences in LAB composition ($P \leq 0.001$) (Fig. 2C). In faeces, LAB isolates resistant to 3 antimicrobial agents predominated (25.0 %), followed by those resistant to 4 antimicrobial agents (21.9 %), since the predominant *E. hirae* was mainly resistant to 2 or 3 antimicrobial agents. Other minor species, such as *L. brevis* or *Streptococcus* sp., were principally resistant to 3 or 4 individual compounds. In raw ewe milk, resistance to 4 antimicrobial agents predominated (34.1 %), followed by resistance to 3 antimicrobial agents (20.5 %), since the predominant species, *E. faecalis, B. thuringiensis, and E. hirae,* and most minor species were mainly resistant to 4 and, to a lesser extent, to 3 antimicrobial agents. In whey, a similar trend compared to milk was observed, although resistance to 3 antimicrobial agents predominated (31.6 %), followed by resistance to 4 antimicrobial agents (28.9 %). In this case, the predominant *B. cereus* was equally resistant to 3 or 4 antimicrobial agents, while *Bacillus* sp. was mainly resistant to 4 antimicrobial agents, *E. hirae* was resistant to 3 antimicrobial agents, and most minor species were resistant to 3 or 4 antimicrobial agents. In fresh cheeses, resistance to 4 antimicrobial agents was dominant (26.8 %), followed by resistance to 1 antimicrobial agent (22.0 %). The predominant *Bacillus* sp. species were equally resistant to 3 or 4 antimicrobial agents, while other dominant species such as *E. hirae* being mainly resistant to 1 antimicrobial, *E. faecalis* to 2 antimicrobial agents and *L. lactis* to 4 antimicrobial agents. A large proportion of the minor species were also resistant to 1 or 4 antimicrobial agents. Finally, in the ripened cheeses, resistance to 2 or 3 antimicrobial agents predominated (25.0 % in both cases), since the predominant *Lactobacillus* sp. and *L. paracasei* were resistant to 2 antimicrobial agents, and, to a lesser extent, other minor important species (*B. cereus* or *Bacillus* sp.) were resistant to 3 antimicrobial agents. Overall, the proportion of LAB resistant to 1 or 2 antimicrobial agents increased throughout the production chain, while the proportion of strains resistant to 3 or 4 antimicrobial agents decreased. No differences in terms of abundance were

observed for the susceptible bacteria throughout the production chain ($P > 0.05$), although the bacteria differed taxonomically. In faeces, raw ewe milk and whey, susceptible LAB belonged, mainly, to *E. hirae* and/or *E. faecalis* species, while in fresh cheese, they were, mainly, unidentified *Bacillus* species; and in ripened cheeses, they corresponded to *L. paracasei;* and, to a lesser extent, to *L. plantarum* subsp. *plantarum* and *Lactobacillus* sp.

Regarding individual compounds, the resistance against polymyxin B was the most common (67.0 %), followed by benzylpenicillin (54.7 %) and amoxicillin (51.2 %), and being clearly more sensitive to dihydrostreptomycin (37.4 %) (Table 1, Fig. 2D-E). The predominance of resistance to polymyxin B was observed in all the samples except for ripened cheeses, where a higher prevalence of LAB isolates resistant to benzylpenicillin was observed. Resistance to benzylpenicillin was primarily observed in ripened cheeses, while amoxicillin resistance was mainly detected in faeces and whey, and dihydrostreptomycin resistance was mainly detected in raw ewe milk and fresh cheeses (Fig. 2D-E). This differentiation along the production chain was related to the LAB composition of each sample type ($P \leq 0.05$) (Table 1). *E. durans* or *Lactococcus* sp., followed by *E. faecium,* were the most resistant to dihydrostreptomycin (Fig. 2A), and the most sensitive species were *B. anthracis, E. avium, E. mundtii*, *L. plantarum* or *Streptococcus* sp. In the case of benzylpenicillin, *L. plantarum* subsp. *plantarum, E. durans, Lactococcus* sp. and *Streptococcus* sp. were the most resistant, while *B. anthracis* or *E. avium* were the most sensitive. *E. durans, Lactococcus* sp., *L. lactis* subsp. *lactis* and *Streptococcus* sp. were the most resistant to polymyxin B, while *E. avium* and *L. plantarum* subsp. *plantarum* were the most susceptible. Finally, *E. durans, E. mundtii* or *Streptococcus* sp. were the most resistant to amoxicillin, and *B. anthracis, E. avium* or *L. plantarum* were the most sensitive.

Regarding the AMR profiles of LAB (Fig. 3A-B), which results from all possible combinations of all antibiotics tested, resistance to all antibiotics predominated (24.6 %), followed by polymyxin B-benzylpenicillin-amoxicillin (16.7 %), polymyxin B (6.90 %) and dihydrostreptomycin-polymyxin B (5.42 %). These patterns were mainly observed for *E. hirae* and, to a lesser extent, for *Bacillus* sp., *Bacillus cereus* and

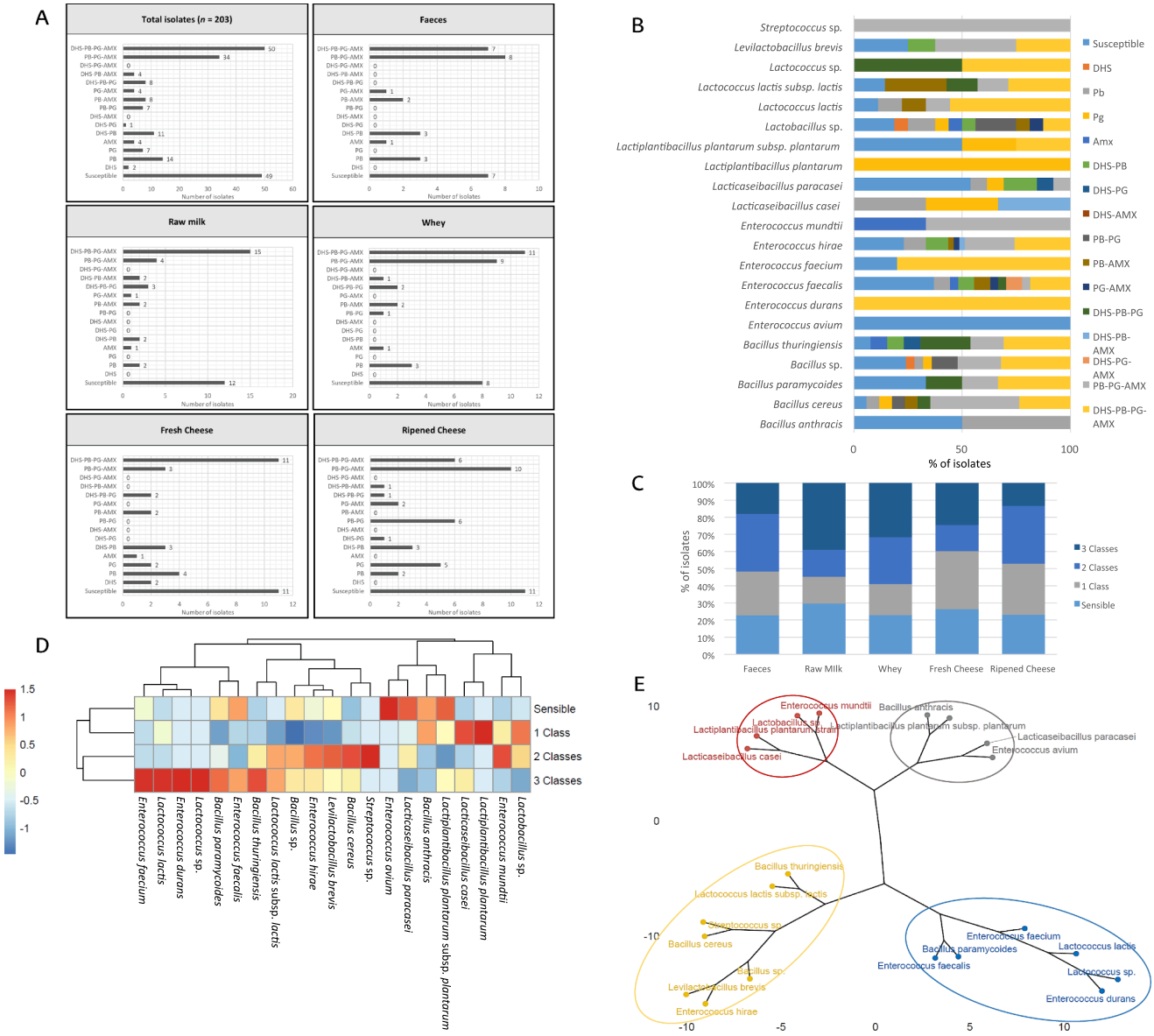

Fig. 3. Box plot representations based on the resistance patterns according to the sample type along production chain and bacterial species (A and B, respectively), and box plot representation (C), HCA heatmap (D) and dendogram clustering (E) based on the resistance against the antimicrobial classes tested according to the sample type along production chain (C) and bacterial species (D and E). Abbreviations: DHS: dihydrostreptomycin; PB: polymyxin B; PG: benzylpenicillin; AMX: amoxicillin.

E. faecalis. Resistance patterns were significantly related to the LAB communities ($P \leq 0.01$), consequently leading to differentiation in the cheese production chain. Resistance to all antibiotics was mainly observed for *E. hirae* and *Bacillus* sp., followed by *L. lactis* and *E. faecalis*, while the pattern of resistance to polymyxin B-benzylpenicillin-amoxicillin was most common for *E. hirae*, *B. cereus* and *Bacillus* sp. (Table 1, Supplementary Fig. 2). Resistance to polymyxin B was related to *E. hirae*, *E. faecalis* and *Lactobacillus* sp., and dihydrostreptomycin-polymyxin B combination was observed in *E. hirae*, *E. faecalis* and *L. paracasei* (Table 1, Supplementary Fig. 2). Thus, considering LAB communities throughout the production chain, resistance to polymyxin B-benzylpenicillin-amoxicillin (25.0 %) and to all antibiotics predominated (21.9 %) in faeces. In raw ewe milk, a similar trend was observed, although resistance to all antimicrobials was notably greater (34.1 %) than that to polymyxin B-benzylpenicillin-amoxicillin (9.09 %), which was maintained in whey (28.9 % and 23.7 %, respectively) and fresh cheeses (26.8 % and 7.32 %, respectively), albeit at different proportions. In the ripened cheeses, the resistance to all the antimicrobial agents was clearly lower (12.5 %), and the resistance to polymyxin B-benzylpenicillin and benzylpenicillin was also notable (12.5 % and 10.4 %, respectively). Notably, no isolate resistant to the combination of dihydrostreptomycin-amoxicillin or dihydrostreptomycin-benzylpeni cillin-amoxicillin was found in any type of sample.

In relation to multidrug resistance (MDR) (Fig. 3C-E), which is defined as resistance to 3 or more classes of antibiotics, it was observed in 30.5 % of the LAB isolates. The MDR differed throughout the production chain ($P \leq 0.05$). Specifically, the proportion of bacteria resistant to 1 class of antimicrobial did not differ throughout the production chain (12.9–29.0 %) ($P > 0.05$). However, the number of isolates resistant to 2 classes differed significantly according to the sample type ($P \leq 0.05$), with the highest prevalence found in ripened cheeses (32.8 %) and faeces (21.3 %) and the lowest in raw ewe milk and fresh cheeses (13.1 % in both cases) (Fig. 3C). The prevalence of multirresistant bacteria also differed throughout the production chain ($P \leq 0.01$), with the highest rate observed in raw ewe milk (32.3 %), which decreased throughout cheese-making and ripening processes (12.9 %) (Fig. 3C). These dynamics were related to the LAB communities ($P \leq 0.05$) (Supplementary Fig. 3), since MDR was mainly observed in *E. hirae* (17.2 %), *E. faecalis* (15.6 %), *B. thuringiensis* (15.6 %) and *Bacillus* sp. (15.6 %). Resistance to 2 classes was detected mainly in *E. hirae* (23.7 %), *B. cereus* (15.3 %) and *Bacillus* sp. isolates (13.6 %), and resistance to 1 class was more common in *Lactobacillus* sp. (19.4 %) and *E. hirae* (16.1 %). Moreover, the HCA and dendrogram divided LAB species into 4 clusters according to the predominant phenotype within each species. Thus, the MDR phenotype was predominant within *E. durans*, *Lactococcus* sp., *E. faecium, L. lactis, B. thuringiensis* and *B. paramycoides*.

3.3. Genotypic profile of antimicrobial resistance

Regarding the ARGs and MGEs, 37 out of the 47 genes studied were detected (Fig. 4A, Supplementary Table 3). Among all the samples, the predominant ARGs were *Str* (average relative abundance of 387), followed by *Str*B (39.3) and *aad*A-01 (19.3), while among the MGEs, *tnp*A-02 and *tnp*A-01 predominated (71.3 and 26.5, respectively). The ARGs *aph*, *aph*6ia, *bla*Z, *bla*TEM, *bla*GES, *bla*CTX-M−03, *bla*OKP and *pbp*2x and the MGEs intI1 and tnpA-03 were not detected. In general, aminoglycoside ARGs presented greater abundances than β-lactam, polymyxin and multidrug ARGs. Among the antimicrobial agents, the *Str* gene exhibited the highest relative abundance within aminoglycosides, followed by *Str*B and *aad*A-01, while *aac*A/*aph*D and *aad*A5-01 presented the lowest abundances. For β-lactams, *bla*-ACC-1, *pbp* and *bla*CMY2-01 dominated, while *amp*C, *bla*IMP-01 and *pbp*5 were minor ARGs. For polymyxins, *mcr*-2 presented the greatest abundance compared to *mcr-1*. Among the multidrug ARGs, two genes were also detected, namely, *tol*C-01 and *mex*D, the first presenting the greatest abundances. Among the MGEs, transposons presented the greatest abundance, especially *tnp*A-02 and *tnp*A-01, while *tnp*A-07 was the least abundant. A similar abundance was observed for integrons, namely, *int*I and IS613. Overall, predominant transposons presented higher abundances than predominant aminoglycoside ARGs, with the exception of *Str*, while lower abundances were observed for integrons, similar to β-lactams, polymyxins and multidrug ARGs.

PERMANOVA indicated that there were no differences among producers in terms of the detected ARGs or MGEs ($P > 0.05$). Nonetheless,

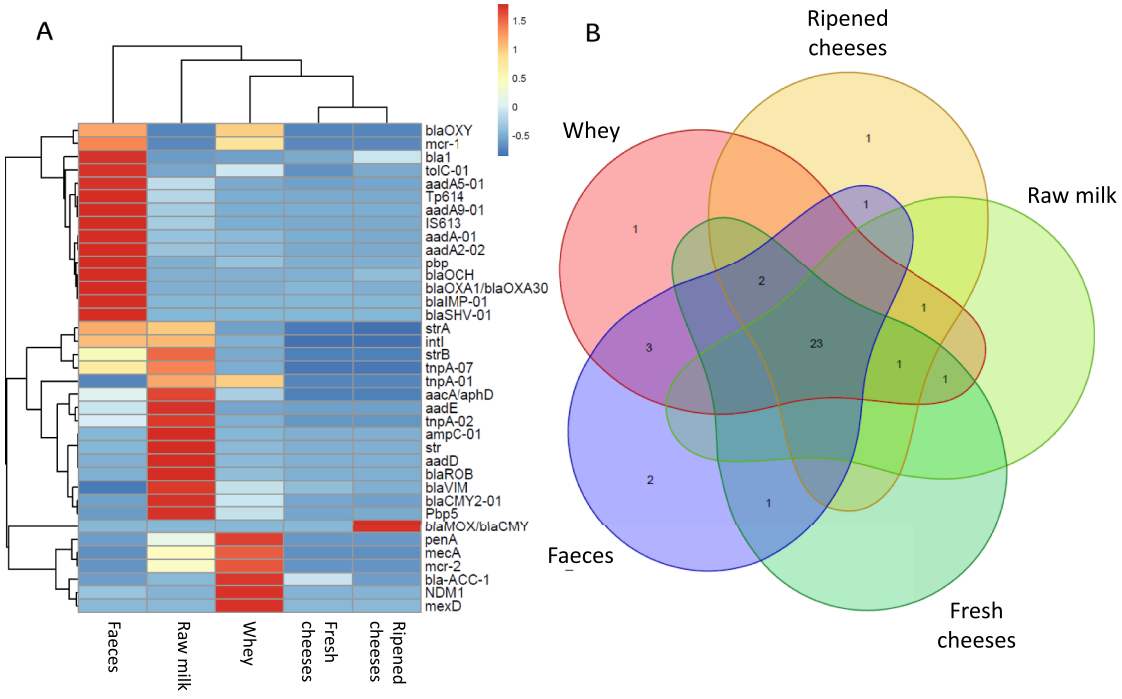

Fig. 4. HCA heatmap (A) and Venn diagram (B) showing the distribution of ARGs and MGEs along cheese production chain (faeces, raw milk, whey, fresh cheeses and ripened cheeses).

there was clear differentiation among the sample types along the production chain ($P \leq 0.001$), which was consistent with the phenotypic results. Specifically, significant differences were detected for all ARGs and MGEs among sample types, except for *aad*A5-01, *aad*D, *bla*IMP-01, *bla*MOX/*bla*CMY, *bla*OXA1/*bla*OXA30, *bla*ROB, *bla*VIM and *mex*D ($P > 0.05$) (Supplementary Table 3). As shown in Fig. 4B, the greatest number of ARGs and MGEs was observed in faeces and whey (32), followed by fresh and ripened cheeses (28 and 29, respectively) and, finally, raw ewe milk (26). However, considering their abundance, the raw ewe milk samples presented the greatest abundance of ARGs and MGEs, followed by the faeces and whey samples, while the fresh and ripened cheese samples presented the lowest abundances ($P \leq 0.001$) (Fig. 4A). Among the predominant ARGs, the greatest abundance of *Str* and *Str*B was observed in raw ewe milk, followed by whey ($P \leq 0.001$); and for *aad*A-01, the greatest abundance was observed in raw ewe milk, followed by whey and faeces ($P \leq 0.001$). For *bla*-ACC-1 and *pbp*, the greatest abundance was observed in raw ewe milk ($P \leq 0.01$), although it did not differ from that in fresh or ripened cheeses or whey. Similarly, *bla*CMY2-01 dominated in whey and fresh cheeses ($P \leq 0.001$). For *mcr*-2, the greatest abundance was observed in whey ($P \leq 0.05$), and for multidrug ARGs, the greatest abundance of *tol*C-01 was observed in faeces ($P \leq 0.05$). In terms of MGEs, the greatest abundance of *tnp*A-02 was observed in raw ewe milk and whey ($P \leq 0.001$), while for *tnp*A-01 and *int*I, it was observed in raw ewe milk ($P \leq 0.01$), and for IS613, it was observed in faeces ($P \leq 0.001$). Overall, the differences in the genotypic profiles of ARGs and MGEs among sample types and the lack of differentiation among producers were confirmed by PCA and OPLS-DA models (Supplementary Fig. 4).

As shown in Fig. 4B, a Venn diagram revealed that the detection of 14 ARGs and MGEs differed among sample types; these ARGs were mainly β-lactam ARGs (11) and, to a lesser extent, polymyxin ARGs (2) and multidrug ARGs (1). Specifically, *bla*IMP-01 and *NDM*1 were exclusively detected in faeces, *mex*D in whey and *bla*MOX/*bla*CMY in ripened cheese samples. The rest were detected in more than one sample type; for example, *mcr*-1, *bla*VIM and blaOXY were detected only in faeces and whey, while *bla*ROB was detected in milk, whey and fresh cheeses.

4. Discussion

Historically, studies on AMR have focused predominantly on pathogenic bacteria, primarily because of their direct implications for human health. However, recent studies have shifted their focus to non-pathogenic bacteria since they can act as AMR reservoirs that can be transferred to pathogens (Wolfe, 2023). In this context, the food and food production chain have been proposed as possible vehicles for the dissemination of AR bacteria and ARGs (Caniça et al., 2019; Wang et al., 2006; Yasir et al., 2022). Hence, the study of raw fermented foods holds particular significance due to their greater bacterial density, which could reach the gastrointestinal tract and interact with human microbiota transferring genes (Abriouel et al., 2015). Consequently, they may pose a significant risk, particularly whether ARGs are transferred to pathogens (Wolfe, 2023).

LAB predominate in fermented products, contributing to flavour and texture development, preventing the proliferation of pathogenic and spoilage bacteria (Fox et al., 2017; Santamarina-García et al., 2023) and exhibiting probiotic effects (Santamarina-García et al., 2020; Wolfe, 2023). However, studies on AMRs in LAB are limited to a certain product, such as milk or cheese (Výrostková et al., 2020, 2021), and to a certain LAB genera, such as *Lactobacillus* (Shi et al., 2023; Štšepetova et al., 2017). Therefore, this work aimed to shed light on the occurrence of AMR in LAB in ovine faeces, raw ewe milk, whey, fresh and ripened cheeses. To date, no information has been published in the literature in this regard.

The results revealed significant variations in LAB prevalence throughout the production chain. Higher LAB counts were observed in faeces than in raw ewe milk; which subsequently increased during whey production and, ultimately, in fresh and ripened cheeses. Although culture-dependent methods have been widely employed to characterize bacterial prevalence (Yap et al., 2022), to the best of our knowledge, no previous study has reported the evolution of LAB prevalence from animals, in this case, sheep, to the final transformed product, namely, ripened cheese. Previous research has focused primarily on the transition from raw milk to fresh and ripened cheeses, although information on raw ewe milk cheeses is scarce (Feutry et al., 2012; Navidghasemizad et al., 2009; Pérez-Elortondo et al., 1993, 1998, 1999). In fact, there are no results in the literature about the prevalence of LAB in ovine faeces and they are limited for whey (Gaglio et al., 2019; Blaiotta et al., 2021). Overall, based on the results obtained, there are notable variations in LAB counts in raw ewe milk or derived cheeses compared to those in other studies (Blaiotta et al., 2021; Khaldi et al., 2022; Rocha et al., 2023), due to factors such as breed, flock management and feeding, sources of microorganisms, for instance, diseases or the dairy environment, or practices followed by producers during milking or cheese-making, for example (Bokulich & Mills, 2013; Esteban-Blanco et al., 2020; Floridia et al., 2023; Fox et al., 2017; Jia et al., 2023; O'Sullivan & Cotter, 2017; Possas et al., 2021; Sun et al., 2019). The observed differences in LAB counts among the producers involved in this study were not statistically significant for all the samples, suggesting that they followed similar flock management and cheese-making practices, contrary to what has been reported in previous studies (Aldalur et al., 2019; Santamarina-García et al., 2022a). Nevertheless, there is no information in the literature on the similarities or differences in LAB counts among producers of the same type of cheese.

The LAB isolates obtained along the raw ewe milk cheese production chain were identified by sequencing the V1-V3 hypervariable region of the gene encoding 16S rRNA. This region was selected because of its great capability and accuracy in providing reliable taxonomic identification (Winand et al., 2019). All the LAB isolates belonged to the Firmicutes phylum, the class Bacilli and the orders Lactobacillales and Bacillales, as expected (Erkmen, 2022). Moreover, four LAB families were identified, namely, *Bacillaceae*, *Enterococcaceae*, *Lactobacillaceae* and *Streptococcaceae*, and eight genera, *Bacillus, Enterococcus, Lactobacillus, Lacticaseibacillus, Lactiplantibacillus, Lactococcus, Levilactobacillus* and *Streptococcus*. The LAB composition along the production chain partially agrees with the findings of previous studies on Idiazabal cheese (Pérez-Elortondo et al., 1993, 1998, 1999) and other raw ewe milk cheeses (Blaiotta et al., 2021; Rocha et al., 2023), since there are differences in the identified LAB and their abundances. For example, *Leuconostoc* species have previously been identified along Idiazabal cheese and other raw ewe milk cheeses production chains, which have not been detected in this study (Pérez-Elortondo et al., 1993, 1998, 1999; Blaiotta et al., 2021; Rocha et al., 2023). Overall, the observed differences could be related to factors such as animal breed, flock management and feeding, sources of microorganisms or practices followed by producers during milking or cheese-making, as mentioned above (Abriouel et al., 2017; Bokulich & Mills, 2013; Esteban-Blanco et al., 2020; Floridia et al., 2023; Fox et al., 2017; Jia et al., 2023; O'Sullivan & Cotter, 2017; Possas et al., 2021; Sun et al., 2019). The discrepancy between these results and those of high-throughput sequencing (HTS) studies on Idiazabal cheese and other raw ewe milk cheeses should be highlighted (Cardinali et al., 2021; Dimov et al., 2021; Santamarina-García et al., 2022a). For instance, in our previous study, *Lactococcus* was identified as one of the dominant genera in raw milk from Latxa sheep and the dominant genus in Idiazabal cheese (Santamarina-García et al., 2022a). HTS techniques enable the detection of numerous bacteria, even those present at relatively low abundances (Abriouel et al., 2008; Michailidou et al., 2021; Santamarina-García et al., 2022a). However, the viability of these organisms cannot be determined, underscoring the necessity of complementing culture-dependent methods (Ferrocino et al., 2022).

The main antimicrobial agents used on sheep farms under the Idiazabal PDO include aminoglycosides, specifically dihydrostreptomycin; β-lactams, such as benzylpenicillin and amoxicillin; and polymyxins,

specifically polymyxin B; which have been previously reported as some of the most widely used antibiotics for livestock and, particularly, sheep (Virto et al., 2022). The presence of antimicrobial agents along the production chain exerts significant selective pressure, contributing significantly to the emergence of AR bacteria (Ammor et al., 2007; Virto et al., 2022; Wolfe, 2023). In fact, when bacterial populations, such as LAB, are exposed to these agents, they are prone to developing resistance (Virto et al., 2022). Subsequently, they can transfer their AMR genes to other bacteria, including pathogens that might pose a health risk (Abriouel et al., 2017; Nunziata et al., 2022; Virto et al., 2022). Hence, it is essential to investigate the resistance of microbial communities to the most commonly used antibiotics (Mathur & Singh, 2005; Wolfe, 2023). No study has analysed the antibiotic susceptibility of LAB in sheep and throughout the production of raw sheep milk cheeses. In fact, there are no data on the antibiotic susceptibility of LAB isolated from sheep, while information on raw ewe milk or raw ewe milk cheeses is scarce (Chen et al., 2020; Kmet' & Drugdová, 2012; Nalepa & Markiewicz, 2023; Rajput et al., 2022; Tsigkrimani et al., 2022), and there are also no data on the whey generated in the production of raw ewe milk cheese. In order to compare with LAB isolates obtained from other livestock or derived foods, the diverse approaches employed, as well as interpretations of the results, hamper comparing studies of the literature. Thus, this study followed international standardized methods to determine MICs (ISO/IDF, 2010; Rychen et al., 2018), namely, by the broth microdilution method and using ECOFFs established by either the EUCAST (https://www.eucast.org) or the EFSA (Rychen et al., 2018).

Out of the more than 200 LAB isolates collected during the Idiazabal cheese production chain, more than 75.0 % were resistant, primarily to polymyxin B, and were notably more susceptible to dihydrostreptomycin. Notably, there is no information on the resistance to polymyxin B or amoxicillin of LAB isolates obtained from faeces, raw milk, whey, curd and cheese from any sheep breed. Resistance against streptomycin has been proven for LAB isolates obtained from raw ewe milk and Feta and Kefalograviera cheeses, and even if the number of LAB isolates tested was lower, most of them were resistant, which supposes higher rates than those obtained in this study (Rajput et al., 2022; Tsigkrimani et al., 2022). For benzylpenicillin, Chen et al. (2020) have reported that all LAB isolates from sheep milk were susceptible, similar to the findings of Kmet' & Drugdová (2012) for ovine cheese isolates, which would not agree with these results. Compared to other studies (Nunziata et al., 2022; Wang et al., 2018; Zhou et al., 2005), D'Aimmo et al. (2007) have also reported a greater MIC for polymyxin B than for dihydrostreptomycin and, to a lesser extent, than for benzylpenicillin for LAB isolated from dairy products. Vidal & Collins-Thompson (1987) have reported a greater prevalence of LAB resistant to dihydrostreptomycin than to polymyxin B or benzylpenicillin, and Gad et al. (2014) have also reported a similar resistance of LAB to benzylpenicillin and amoxicillin, slightly predominating resistance to benzylpenicillin. The differences compared to previous studies are due to the different LAB obtained (D'Aimmo et al., 2007; Gad et al., 2014; Guan et al., 2017; Nunziata et al., 2022; Vidal & Collins-Thompson, 1987; Wang et al., 2018; Zhou et al., 2005). Nevertheless, while LAB have traditionally been considered resistant to aminoglycosides and susceptible to β-lactams, an increasing number of LAB isolates resistant to β-lactams have been obtained in recent years (Abriouel et al., 2015; Ammor et al., 2007; Devirgiliis et al., 2008; Nunziata et al., 2022), agreeing with the obtained results. This could be related to the selective pressure exerted by antibiotic utilization and the transfer of ARGs (Nunziata et al., 2022; Virto et al., 2022).

Taxonomically, *Lactococcus* species, such as *L. lactis* and *L. lactis* subsp. *lactis*, which play important roles in technological processes such as cheese production (Nunziata et al., 2022), were some of the most resistant LAB. No information on the resistance to polymyxin B or amoxicillin of *Lactococcus* species isolated from ovine faeces, raw ewe milk, whey or raw ewe milk cheeses has been reported. Regarding benzylpenicillin, Chen et al. (2020) have reported that all *L. lactis* isolated from Hu sheep milk were sensitive, which do not agree with these results. For streptomycin, Tsigkrimani et al. (2022) have reported that all *L. lactis* isolates obtained from sheep milk and artisanal Feta and Kefalograviera cheeses were resistant, in line with these results. Overall, *Lactococcus* species are considered resistant to aminoglycosides, such as streptomycin (Ammor et al., 2007; Nunziata et al., 2022; Sharma et al., 2014), and polymyxins, specifically polymyxin B (Khemariya et al., 2013); however, they are reportedly sensitive to β-lactams, including benzylpenicillin and amoxicillin (Ammor et al., 2007; Nunziata et al., 2022; Sharma et al., 2014). However, recently, β-lactam-resistant *L. lactis* strains have been reported (Kazancıgil et al., 2019), which corroborates the resistance rates observed in this study. In addition, in the present study a distinction among species was observed, with *L. lactis* and *L. lactis* subsp. *lactis* being more sensitive to dihydrostreptomycin, while unidentified *Lactococcus* species were more susceptible to amoxicillin. No similar results have been reported to date (Nunziata et al., 2022).

The unidentified *Streptococcus* species also exhibited high resistance rates. Notably, information on the resistance of *Streptococcus* species isolated from ovine faeces, raw ewe milk, whey or raw ewe milk cheeses to the tested antimicrobial agents is limited in the literature. In general, high but variable resistance to β-lactams, including benzylpenicillin, has been reported (Ammor et al., 2007; Flórez & Mayo, 2017; Morandi et al., 2015; Nunziata et al., 2022), in agreement with the obtained results. Information is also scarce for amoxicillin; although resistant strains of *S. agalactiae* isolated from raw goat milk have been identified (Shi et al., 2023), but other species, such as *S. dysgalactiae* or *S. uberis*, from dairy cows have been reported to be sensitive (Dyson et al., 2022). Regarding polymyxin B, there is little information on LAB isolated along the dairy production chain, but strains of *S. thermophilus* have also been reported to be resistant in the literature (Sozzi & Smiley, 1980), which would agree with the results of this study, although there are contradictory results (Rüegsegger et al., 2014). For aminoglycosides, moderate-high resistance has been reported, including streptomycin (Ammor et al., 2007; Nunziata et al., 2022), for which an upwards trend has been reported in recent years (Nunziata et al., 2022; Tosi et al., 2007), which was not observed in the present study.

Within the *Enterococcus* genus, several species presented high levels of resistance, namely, *E. durans* and *E. faecium* and, to a lesser extent, *E. hirae* and *E. mundtii*, which were, in general, mainly resistant to polymyxin B and amoxicillin and, to a lesser extent, to benzylpenicillin. Information on resistance to polymyxin B in *Enterococcus* species obtained from ovine faeces, raw ewe milk, whey or raw ewe milk cheeses has not been reported to date, while for β-lactams and streptomycin is limited. Tsigkrimani et al. (2022) have reported that all *E. faecalis* isolates and more than 70 % of *E. faecium* isolates obtained from raw sheep milk and Artisanal Feta and Kefalograviera cheeses were resistant, similar to the results obtained for dihydrostreptomycin in the present study; whereas Rajput et al. (2022) have also reported an *E. hirae* isolate obtained from raw sheep milk to be resistant. Enterococci are part of the microbiota of many cheeses and contribute to modulating the microbiota, especially pathogenic and spoilage bacteria, due to their antimicrobial activity and to the development of aroma, flavour and texture (Dapkevicius et al., 2021; Franz et al., 2011; Santamarina-García et al., 2023; Santamarina-García et al., 2022b). However, these strains are not classified as qualified presumption of safety (QPS), as they present virulence genes and ARGs that cause them to commonly act as opportunistic pathogens (Dapkevicius et al., 2021; Franz et al., 2011). *Enterococcus* species have previously been described as resistant to β-lactams, including benzylpenicillin and amoxicillin, or to aminoglycosides, such as streptomycin (Dapkevicius et al., 2021; Franz et al., 2011). *E. faecium* and *E. faecalis* are considered the most important species in terms of AMRs (Dapkevicius et al., 2021), although there are notable differences in the literature (Dapkevicius et al., 2021; Franz et al., 2011; Gaglio et al., 2016; Gołaś-Prądzyńska et al., 2022; Juliano et al., 2022). Juliano et al. (2022) have reported similar resistance

against amoxicillin among *E. faecalis, E. faecium, E. hirae* and *E. mundtii,* and higher resistance against benzylpenicillin for *E. hirae* than for *E. faecalis* and *E. faecium* isolated from milk and dairy environments. Gołaś-Prądzyńska et al. (2022), instead, have not isolated benzylpenicillin or streptomycin-resistant *E. faecalis* or *E. faecium* from raw ewe milk and derived cheese. Information on the resistance of *Enterococcus* species to amoxicillin in livestock and dairy products is scarce, and only Bag et al. (2022) have reported that all *E. faecalis* isolates obtained from cow milk were sensitive. To the best of our knowledge, there is no information on polymyxin B.

Different species of the *Bacillus* genus have been studied for their potential use as probiotics, as their interest resides in the ability of spores to resist heat and gastric pH (Amoah et al., 2021; Santamarina-García et al., 2020). However, in addition to pathogenicity, AMRs should also be studied (Amoah et al., 2021; Sharma et al., 2014). In this study, most *Bacillus* species, such as *B. cereus, B. paramycoides, B. thuringiensis* or *Bacillus* sp., exhibited high resistance rates, specifically to polymyxin B, benzylpenicillin and amoxicillin and, to a lesser extent, to dihydrostreptomycin. Information on the resistance of *Bacillus* species isolated from ovine-derived faeces, raw ewe milk, whey or cheeses to the tested antimicrobial agents is scarce. In general, a high resistance of *Bacillus* species resistant to benzylpenicillin has been reported before in dairy environments and products (Bartoszewicz & Czyżewska, 2021; Gao et al., 2018; Owusu-Kwarteng et al., 2017; Zhai et al., 2023), albeit at different rates. For instance, Bartoszewicz & Czyżewska (2021) have reported that the 98.9 % of *B. cereus* isolates and 100 % of *B. thuringiensis* isolates obtained from raw cow milk and dairy environments were resistant, while Al-harbi et al. (2021) have found that only 52 % of the *Bacillus* sp. isolated from cow milk were resistant. For amoxicillin, information is scarce, but Owusu-Kwarteng et al. (2017) have reported that 100 % of *B. cereus* isolates obtained from dairy environments, milk and dairy products were resistant to amoxicillin. Information on susceptibility to streptomycin is also scarce, but most studies have reported *Bacillus* strains obtained from dairy cows, raw milk and dairy environments to be susceptible (Cui et al., 2016; Liu et al., 2022), which would corroborate the lowest resistance observed in this study compared to the other antimicrobial agents tested. Finally, a unique work has evaluated the polymyxin susceptibility of *Bacillus* species (Amoah et al., 2021), and, specifically, there is no information on dairy products. Amoah et al. (2021) isolated *B. tequilensis, B. velezensis* and *B. subtilis* species from the intestine of hybrid grouper, all of which were susceptible to polymyxin B.

The different species of the genus *Lactobacillus*, along with other species that have traditionally belonged to this genus but have recently been reclassified, specifically, *Lacticaseibacillus*, *Lactiplantibacillus* and *Levilactobacillus* (Zheng et al., 2020), presented lower resistance to antibiotics, except for *Levilactobacillus* isolates. Overall, no information has been reported regarding the resistance of *Lactobacillus* species isolated from ovine-derived faeces, raw ewe milk, whey or raw ewe milk cheeses to polymyxin B or β-lactams. For streptomycin, there is little information available, but Tsigkrimani et al. (2022) have recently reported that all *L. plantarum* isolates and more than 90 % of *L. brevis* isolates obtained from raw sheep milk and Artisanal Feta and Kefalograviera cheeses were resistant, which means higher resistance compared to the results of the present study. Traditionally, *Lactobacillus* species have exhibited low AMR, although greater variability has been reported in recent years (Abriouel et al., 2015; Ammor et al., 2007; Devirgiliis et al., 2008; Nunziata et al., 2022). In general, the number of currently reported lactobacilli-related infections is quite low, and there is no evidence of opportunistic infection by lactobacilli from fermented foods (Abriouel et al., 2015). Nonetheless, the acquired antimicrobial resistance of *Lactobacillus* to different antibiotics has been previously demonstrated (Abriouel et al., 2015; Mathur & Singh, 2005; Sharma et al., 2014). Furthermore, if *Lactobacillus* species act as reservoirs of AMRs, they could pose a threat to human health, especially if fermented foods containing antibiotic-resistant *Lactobacillus* are consumed in substantial quantities and if resistance genes are transferred to intestinal bacteria (Abriouel et al., 2015; Ammor et al., 2007; Nunziata et al., 2022). Moreover, different *Lactobacillus* species and strains have been widely used as probiotics, but previously must be classified as QPS, for which antimicrobial resistance profile is required (Sharma et al., 2014). Consequently, there are many studies in the literature on the AMRs of *Lactobacillus* species (Abriouel et al., 2015, 2017; Mathur & Singh, 2005). In the present study, the *Levilactobacillus* genus and the *L. brevis* species were the only of all those species previously classified as *Lactobacillus* that presented moderate-high resistance, especially to polymyxin B and β-lactams, and were more sensitive to the aminoglycoside dihydrostreptomycin, in agreement with previous works (Ammor et al., 2007; Nunziata et al., 2022). Nonetheless, great variability in the antimicrobial resistance profile among *Lactobacillus* species has been observed (Mathur & Singh, 2005; Nunziata et al., 2022), and there is no clear pattern of resistance according to species (Nunziata et al., 2022). In general, *Lactobacillus* species tend to be intrinsically resistant to aminoglycosides such as streptomycin, which is not observed in all *Lactobacillus* isolates obtained along the Idiazabal cheese production chain; however, for β-lactams, such as against benzylpenicillin and amoxicillin, are reportedly sensitive (Muñoz et al., 2014; Happel et al., 2020; Nunziata et al., 2022). Nonetheless, recent studies have highlighted a greater resistance to benzylpenicillin, for example, in *L. plantarum* or *L. casei* (Abriouel et al., 2015; Majhenič et al., 2007; Nunziata et al., 2022), in line with the results of this study. According to amoxicillin, although few isolates were resistant, particularly *Lactobacillus* sp. and *L. casei*, non-resistant *Lactobacillus* species have been reported to date in the literature (Muñoz et al., 2014; Happel et al., 2020). There is little information on the resistance of *Lactobacillus* species to polymyxin B (Ruiz-Moyano et al., 2019). Ruiz-Moyano et al. (2019) have recently reported that all *L. brevis, L. casei* and *L. paracasei* strains obtained from Serpa cheese were resistant to polymyxin B and more susceptible to benzylpenicillin, in line with the results of the present study.

It has previously been observed that the bacterial dynamics during the production of raw ewe milk cheeses determine the different quality and safety parameters of cheese, such as biogenic amines or volatile compounds (Santamarina-García et al., 2023; Santamarina-García et al., 2022b). In this sense, the resistance rates and trends observed in each sample along the production chain were also due to the different composition of LAB. Thus, the predominance of *Enterococcus* species, such as *E. hirae*, and *Bacillus* species, such as *B. cereus* or *Bacillus* sp., in ovine faeces and raw ewe milk, as well as *Lactococcus* species, specifically *L. lactis*, in whey and fresh cheese, contributed to the high resistance rates observed. However, the resistance rate of LAB in the final cheese decreased, mainly due to the predominance of *Lactobacillus* sp. and *Lacticaseibacillus* species. Similarly, MDR was also reduced along the production chain due to the inhibition of the main multidrug-resistant species, such as *E. hirae* or *Bacillus* sp. Similarly, the predominant patterns of resistance, namely, all antimicrobial agents and polymyxin B-benzylpenicillin-amoxicillin, were also reduced in ripened cheeses since they were mainly observed in *E. hirae, B. cereus* and *Bacillus* sp., while polymyxin B-benzylpenicillin and benzylpenicillin patterns gained importance related to the greater abundance of *Lactobacillus* species. Therefore, it can be concluded that the production chain modulates the prevalence of AMR in LAB and favours a greater abundance of susceptible LAB. This is of special interest in terms of cheese safety (Virto et al., 2022), considering that ripened cheese presented the highest number of CFU compared to the rest of the samples, specially ovine faeces, raw ewe milk and whey, and is indeed what is consumed and comes into contact with the intestinal microbiota (Wolfe, 2023). Overall, there are no results on resistance patters on MDR along production chain for other cheeses.

Several studies have highlighted the importance of LAB as reservoirs of ARGs, which can be transferred to the human microbiome (Nunziata et al., 2022). To analyse the ARGs that confer resistance to those antibiotics used in dairies, the most important genes for LAB were selected

using the CARD database (Alcock et al., 2023) and subsequently analysed by HT-qPCR (Jauregi et al., 2021). Compared to traditional qPCR, this technique has many advantages, such as greater speed and efficiency, in addition to the large number of genes that can be detected at the same time. Likewise, it has better detection limits than metagenomic sequencing approaches (Waseem et al., 2019). However, this approach also has several disadvantages, such as the inability to analyse unknown sequences since it requires the previous design of primers (Waseem et al., 2019). Notably, although this technique is one of the most appropriate methods for targeting ARGs, it has been applied mainly to environmental samples and not to dairy products (Wei et al., 2022; Yang et al., 2022), which highlights the novelty of this study.

In this study, clear differences were observed in the abundances of ARGs and MGEs throughout the production chain, with the greatest abundances observed in raw ewe milk, followed by ovine faeces and whey, while fresh and ripened cheeses presented the lowest abundances. Overall, these genotypic results would coincide with the phenotypic results obtained, indicating that microbial dynamics during the cheese-making process facilitate the reduction of ARGs and MGEs, which has not been reported so far. Overall, this would make sense since microbial dynamics have also been previously described as responsible for the evolution of other food safety concerns, such as biogenic amines (Santamarina-García et al., 2022b). In different studies, the presence of ARGs and MGEs in animal manure and dairy products, such as milk or cheese, is analysed via qPCR (Delannoy et al., 2023; Kang et al., 2022). However, there are no results using HT-qPCR. Anyway, to date, no study has analysed the prevalence of ARGs and MGEs throughout the dairy production chain using qPCR or HT-qPCR techniques.

The genes with the highest abundance were *Str*, *Str*B and *aad*A-01, which were also the predominant ARGs in terms of resistance against aminoglycosides. Few studies have analysed ARGs related to LAB using qPCR or HT-qPCR techniques (Guo et al., 2020), since most genotyping approaches are based on conventional PCR (Nalepa & Markiewicz, 2023; Obioha et al., 2023), which makes it impossible to determine which genes are the most abundant. The predominance of these genes throughout the production chain in terms of resistance to aminoglycosides is consistent with what has been reported in the literature for LAB obtained from dairy products, although there are differences (Gaglio et al., 2016; Nunziata et al., 2022; Obioha et al., 2023). For instance, Obioha et al. (2023) have detected only the *aad*E gene in LAB from a fermented dairy product. For β-lactams, different ARGs, such as *bla*Z, *amp*C or *mec*A, have been described as predominant (Nunziata et al., 2022; Rosander et al., 2008), most of which were detected in this study. However, there is no information on the predominant genes observed in this study, namely, *bla*-ACC-1, *pbp* and *bla*CMY2-01. On the other hand, the detected *mcr*-1 gene was the first ARG described in the literature for polymyxins (Nagy et al., 2021) and has been detected in livestock faeces and dairy products, although mainly from cows (Chen et al., 2017; Zheng et al., 2019). Up to 11 variants have been described because they are found in a plasmid; therefore, they are easily transmitted between bacteria (Nagy et al., 2021). However, *mcr* genes have been described only for *Enterobacteriaceae* (Chen et al., 2017; Nagy et al., 2021; Zheng et al., 2019), and there are no results related to LAB. Similarly, genes that confer multidrug resistance in LAB have also been described (Nunziata et al., 2022), within which the multidrug ARGs *mex*D and *tol*C were detected. Overall, few genes that confer multiresistance in LAB have been described, although the phenotypic profile has been widely observed, mainly in *Enterococcus*, as in this study (Gaglio et al., 2016; Obioha et al., 2023; Oguntoyinbo & Okueso, 2013). As previously mentioned, ARGs can be transmitted between bacteria by means of MGEs, within which, transposons and integrons predominate (Iskandar et al., 2022; Nunziata et al., 2022). In this study, 6 out of the 7 MGEs studied were detected, predominantly the transposons *tnp*A-02 and *tnp*A-01 and the integrons *int*I and IS613. Overall, information on these MGEs from LAB is scarce, but several integrons and transposons have been reported before (Nunziata et al., 2022).

5. Conclusions

This study provides new insights into the prevalence of AMRs related to LAB along a raw ewe milk cheese production chain (ovine faeces, raw ewe milk, whey, fresh cheese and 60-day-ripened cheese). Both phenotypic and genotypic results revealed a decrease in resistance rates, including patterns and MDR, as well as a reduction in the relative abundance of ARGs and MGEs along the production chain. This was related to the changes in the LAB composition throughout the production chain. Thus, the abundance of those LAB that presented high resistance in ovine faeces, raw ewe milk, whey and fresh cheeses, such as *Lactococcus, Enterococcus* and *Bacillus* species, was notably reduced in the ripened cheeses. In contrast, more sensitive LAB species, such as *Lacticaseibacillus* and *Lactobacillus*, became more abundant. These findings are of special interest, because they indicate the role of the production chain in minimizing AMRs, which has not been reported to date, and the greater susceptibility of those LAB that are consumed with cheese and that come into contact with the intestinal microbiota. However, further research is needed to elucidate the influence of different factors, such as herd management or cheese-making conditions, on the AMRs of LAB to identify the key factors involved in controlling resistant LAB.

CRediT authorship contribution statement

Gorka Santamarina-García: Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gustavo Amores:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Formal analysis, Conceptualization. **Diego Llamazares:** Investigation. **Igor Hernández:** Conceptualization, Methodology, Resources, Writing – review & editing. **Luis Javier R. Barron:** Funding acquisition, Project administration, Resources. **Mailo Virto:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114308.

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